DISCUSSION

Plant tissue culture is one of the most dynamic and promising tool for research in experimental biology for the study of various basic problems in plant physiology, plant biochemistry, etc. Plant cell and tissue cultures have enabled us to increase our knowledge tremendously in many areas. Recent advances in this field have made possible the investigations of growth and differentiation at the levels of tissues and cells. The present research endeavour concentrated on: initiation and establishment of callus cultures of *Nicotiana tabacum* L. var. Anand-2 and *Gossypium hirsutum* L., cv. Sankar-5; the examination of growth responses and accumulation of total and reducing sugars; accumulation of extractable starch; organogenetic responses as influenced by growth substances; the physiological changes during growth of callus; the study of certain enzymes during shoot and root differentiation both in light and dark; and lastly the study of these enzymes in cotton callus tissues which remain unorganised even when cultured on shoot and root differentiating medium of tobacco. An attempt was made to find out what is actually contributing for organogenetic development and what is not, by comparing the changes in enzyme activities in a differentiating tobacco callus and a non-differentiating cotton callus tissue.
A. Initiation and establishment of callus cultures of tobacco and cotton.

Callus was induced from the floral buds of *N. tabacum* L. var. Anand-2 as described in Chapter III, Section A, Expt. 1. The tobacco callus tissue was healthy and fast growing and was maintained in 30 day cultural cycles. Chapter III, Section A, Expt. 2, describes the induction and establishment of callus cultures derived from the anthers of cotton. Katterman *et al.* (1977) described the successful initiation and transfer of viable callus of cotton *G. barbadense* L. using Cleland's reagent, dithiothreitol, a strong antioxidant (Cleland, 1964). They used high levels of auxin in the medium described by Beasley and Ting (1973). On the contrary, Anita Rani and Bhojwani (1976) reported that the stem and hypocotyl segments of both *Gossypium hirsutum* and *Gossypium arboreum* did not callus on White's medium alone; while on Murashige and Skoog's medium supplemented with 1 mg/l kinetin, 1 mg/l NAA and 40 mg/l adenine, 80% of the explants revealed proliferation. In the present study, callus was initiated from the anthers of cotton without using any antioxidants in MS medium supplemented with 2 mg/l each of IAA, NAA and kinetin. The callus was initially brown but in course of subcultures, it turned green and maintained a high rate of growth. Calli of different plant species may vary in their texture, friability and coloration. Stem callus of *Citrus grandis* gave callus strains that were greenish-white, compact, nodular, and slow growing, or that were highly friable and fast growing (Mitra and Chaturvedi, 1972).
Cultural conditions for *Gossypium* spp. have been defined also by Smith and Price (1978). Using high light intensity (9000 Lux) at 30°C, they incorporated high doses of hormones, especially cytokinin (e.g. 10 mg/l of 2 i.p) for prolonged cultures of some cotton species.

**B - I. Hormonal influence on growth and accumulation of total and reducing sugars in callus tissues of *Gossypium hirsutum.***

In the present study, four auxins: IAA, IBA, NAA and 2,4-D and two cytokinins namely kinetin and BAP, and one gibberellin (GA$_3$) were tested for their ability to support callus growth and accumulation of total and reducing sugars.

First the above hormones were tested singly and then auxin-cytokinin, auxin-gibberellic acid and auxin-cytokinin-gibberellic acid interactions were studied. The results are given in Chapter III, Section B-I, Expts. 3-6.

A glance at tables 2 to 5 revealed that anther callus of cotton could grow in the absence of exogenous hormonal supply. Growth on fresh and dry weight basis increased 2.9 folds and 3.3 folds respectively during the course of culture for 4 weeks. Increasing concentrations of native auxin IAA stimulated growth, but at 5 mg/l level it was inhibited. The other three synthetic auxins IBA, NAA and 2,4-D inhibited growth of cotton callus with increase in hormonal concentration from 0.2 mg/l to 5.0 mg/l.
Moyed and Tuli (1968) have demonstrated that low levels of 3-methyleneoxindole - the degradation product of IAA - stimulated growth, whereas its higher concentrations antagonized it. Low 3-methyleneoxindole level can be achieved by either low IAA oxidase activity or by high 3-methyleneoxindole reductase activity. So at higher concentrations of IAA (5 mg/l), it may be that the rate of IAA destruction was high, resulting in low endogenous IAA and high 3-methyleneoxindole levels, which did not favour growth. On the other hand, the reverse situation should hold good for stimulation of growth at low levels of IAA.

In view of the known regulation of cell growth by IAA and the inverse correlation of IAA oxidase content with growth (Galston and Dalberg, 1954), it appears likely that the enzymatic destruction of IAA is important in regulating the amount of growth substance in the plant. Also, monophenols stimulate IAA oxidase activity resulting in low growth values; whereas diphenols inhibit IAA oxidase activity thus stimulating growth (Thimann et al., 1962; Furuya and Galston, 1961; Lee and Skoog, 1965 a, 1965 b; Van overbeek, 1966; Tomaszewski and Thimann, 1966).

Maximum cotton callus growth was obtained with the use of IBA in the medium. This cannot be explained because IBA oxidising enzyme has yet not been worked out fully. The literature at present is also scanty on how it affects the
primary and secondary metabolism of the cells in culture.
Bapat and Narayanaswamy (1977) have reported, on the contrary, that IBA did not influence significantly the growth of *Achras sapota* endosperm cultures.

Growth of cotton callus on NAA medium was almost similar to that of IAA. Increasing concentrations of NAA inhibited growth like that of IBA. NAA is reported to stimulate the invertase activity which will result in better utilization of carbohydrate source i.e., sucrose (Glasziou *et al.*, 1966). It is also known to enhance RNA content by as much as 50% (Leaver, 1966), which would result in increased synthesis of structural and functional proteins. NAA is found to accumulate rapidly in plant tissues and is then metabolized slowly to a series of unidentified derivatives (Glasziou *et al.*, 1968). The optimum level of NAA in the system can thus be maintained resulting in rapid growth of callus.

Compared to other three auxins, 2,4-D showed lesser growth response. This could be explained by the following arguments. Firstly, the degradation of 2,4-D in the plant cells is known to be slow. It was shown to metabolize ten times slower than IAA (Bendana *et al.*, 1965), due to possible lack of 2,4-D oxidizing enzyme system. Secondly, the rate of 2,4-D transport is very slow (McCready, 1963). Thirdly, 2,4-D is known to interfere with glucose metabolism (Bourke
et al., 1962) and to increase its catabolism via pentose phosphate pathway (Humphreys and Dugger, 1957).

In many cases, however, 2,4-D is found more effective than either IAA or NAA or IBA in callus induction (Yeoman, 1973) and in the maintenance of active growth and cell division (Tulecke et al., 1965; Gamborg et al., 1968; Rawal, 1979). Lamport (1964) and Simpkins et al. (1970) demonstrated the effectiveness of 2,4-D at low concentrations in promoting high growth rates and cell separation in sycamore suspension cultures. Growth as measured by increase in fresh and dry weight and total cell number was more rapid in NAA medium than that in 2,4-D medium in callus cultures of *Cucumis melo* (Fadia and Mehta, 1973); but in the case of mesocarp and endosperm cultures of *Achras sapota* (Bapat and Narayanaswamy, 1977) and also in callus cultures of *Arachis hypogaea* (Shailaja Rama Rao, 1978) growth was more on 2,4-D than that on IAA or NAA containing medium.

The indispensibility of kinetin for growth of callus tissues has been demonstrated by Murashige and Skoog (1962) and Steward et al. (1969). Kinetin induced growth is accredited to enhanced RNA and protein synthesis (Skoog et al., 1967; Letham and Williams, 1969; Kende and Gardner, 1976). Kinetin has, further, been reported to have additive effect to that of auxin for growth promotion of spruce and sycamore callus cultures (Steinhart et al., 1961; Digby and
Vareing, 1966). Investigations on a range media (Schenk and Hildebrandt, 1972) have shown that tissues of dicotyledonous plants are generally more dependent on an external cytokinin supply than are tissues of monocotyledonous plants. In the present study kinetin stimulated growth of cotton callus (Table 3). Increasing concentrations of kinetin also increased both fresh and dry weight of the tissues. 6-benzylaminopurine (BAP) at 0.04 to 1.0 mg/l level increased dry weight of the tissues but at higher level (2.0 mg/l) it suppressed growth.

Murashige (1964, 1965) working with tobacco callus, and Nickell and Tulecke (1959) working with various monocots and dicots, found GA_3 to inhibit growth. These results were confirmed in our laboratory earlier when tobacco tissues were cultured on GA_3 medium (Rawal, 1979). Gibberellin is known to influence oxidative or peroxidative enzyme activity resulting in a decrease in IAA degradation. Also it was proposed that gibberellin induced the formation of a compound which inhibits the oxidative degradation of IAA. The evidence for this possibility is, however, extremely tenuous; for (a) such an effect must occur within 24 hours to account for the increases in auxin levels noted (Kuraishi and Muir, 1964; Nitsch and Nitsch, 1959, 1963; and Radiouova and Runkoava, 1963); and because (b) the effect must be a pronounced one since increase in auxin level of upto 200 times have been recorded (Nitsch
and Nitsch, 1963). Watanabe and Stutz (1960) observed an increase in an inhibitor concentration in some tissues under some light conditions following $GA_3$ treatment. In the present study, callus cultures of cotton showed higher growth values at 25 mg/l level, while at 100 mg/l $GA_3$ level growth was much reduced. This inhibition of growth by $GA_3$ at 100 mg/l level is in agreement with the findings of Galston (1959) who reported an increased inhibitor content in pea seedlings due to $GA_3$. However, no change was found in the oxidative activity in dwarf pea stems over a 100-fold concentration range.

Of all the auxin-cytokinin combinations tested, 2 mg/l of IAA and 1 mg/l of BAP with 2% sucrose supported maximum growth (Table 4). Of all the auxin-gibberellin interactions tried maximum growth was supported by NAA + $GA_3$ (Table 5). Though on fresh and dry weight basis, 0.2 mg/l NAA, 1.0 mg/l BAP and 25.0 mg/l $GA_3$ in combination supported maximum growth of all the auxin-cytokinin-gibberellin interactions tried, during the subsequent sub-cultures the growth was found to decline very significantly due to some inexplicable reasons.

Both auxins and cytokinins readily form conjugates in plants. IAA and 2, 4-D can be converted to relatively inactive glucosides or glucosyl esters (Feung, 1975), a phenomenon often occurring in monocot tissue, or to the active amino acid conjugates (Feung et al., 1976) which are
more common in dicot tissue. Similarly cytokinins can form a variety of glucosides, some active and some relatively inactive. More recently amino acid conjugates of cytokinins have also been detected (Wang, 1978). Since such conjugates are formed from many exogenous compounds neither conjugation mechanism can be considered unique to the two plant growth regulators. It is more likely that the pathways represent inactivation or storage mechanisms. The ease of hydrolysis of some of these conjugates has led to the assumption that the free acid or base is responsible for any biological activity observed. Thus conjugation may be a way of preserving the biological activity of the plant growth regulators (Everett et al., 1978). An understanding of the molecular action of auxin and cytokinin is further complicated when note is taken of the interactions in tissue cultures which can occur between these two hormones. The effect of cytokinin on both auxin conjugation (Lau and Yang, 1973) and degradation (Lee, 1974) represent direct evidence for one facet of this interaction. However, there is far more circumstantial evidence which indicates that hormones can drastically alter each others metabolism or may indeed have some common sites of action.

Experiments described in Section B-I clearly indicated that the accumulation of total and reducing sugars in cotton callus cultures was influenced by exogenous hormonal supply. Maximum accumulation of total sugars was supported by
0.2 mg/l IAA in the medium; whereas maximum accumulation of reducing sugars was noticed on 2.0 mg/l IAA (Table 2) (Section B-I. Expt. 3). However, a correlation between accumulation of sugars and callus growth could not be drawn. 2.0 mg/l IBA supported maximum accumulation of total sugars, while 5.0 mg/l IBA gave rise to maximum accumulation of reducing sugars, where the growth was minimum. Of all the auxins tested, IBA supported maximum growth as well as maximum sugar accumulation (Table 2). 0.2 mg/l NAA supported maximum growth and also maximum accumulation of reducing sugars. On the other hand, 2.0 mg/l NAA supported minimal growth, but total sugar accumulation was maximum. Like NAA, 2,4-D also showed maximum growth on 0.2 mg/l, where accumulation of reducing sugars was high. 2,4-D at 1.0 mg/l level, exhibited maximum accumulation of total sugars.

Of the two cytokinins tested KN supported maximum growth, while BAP at 2.0 mg/l level supported maximum accumulation of total and reducing sugars. GA3 at lower concentrations i.e., at 5.0 mg/l supported maximum accumulation of total sugars, but reducing sugars were higher at 50.0 mg/l. A correlation between growth and sugar accumulation was not noticed in any of the above treatments (Section B-I. Expt. 4. Table 3).

Among auxin-cytokinin interactions, 2,4-D (0.2 mg/l)
+ BAP (1.0 mg/l) supported maximum accumulation of total and reducing sugars but growth was minimum on this medium. Though growth was moderate, 2.0 mg/l IAA + 25.0 mg/l GA\textsubscript{3} registered maximum accumulation of total and reducing sugars. Out of the auxin-cytokinin-gibberellin combinations, maximum accumulation of total sugars was noticed on 2,4-D + BAP + GA\textsubscript{3} medium where growth was minimum. On the other hand, maximum accumulation of reducing sugars was seen on NAA + KN + GA\textsubscript{3} medium. Effect of phytohormones on the accumulation of sugars is not worked out extensively in cultured plant cells. However, total and reducing sugar accumulation much depends on enzymatic activities. Amylase and invertase are known to produce reducing sugars and these enzymes are much influenced by phytohormones (Bourke et al., 1962; Humphreys and Dugger, 1957; Wedding et al., 1967; Palmer, 1968; Glaziou et al., 1966; Jacobsen, 1970; Varner and Chandra, 1964). Also the possibility of direct and indirect effects, such as influence of auxin levels on sucrose metabolism cannot be ignored (Wiggans, 1954).

B - II. Influence of carbohydrates on growth and accumulation of total and reducing sugars in callus cultures of G. hirsutum.

Experiments conducted with the callus cultures of cotton to examine the influence of different sugars on growth, morphogenetic response if any, and total and reducing sugar
accumulation, are described in Chapter III, Section B-II, and Expts. 7-9. Though a wide range of different sugars was used in the medium as a source of carbon, no morpho-genetic response was observed in callus cultures of cotton. Different concentrations of mono, di, tri and polysaccharides and also sugar alcohols were used in the basal medium to study their influence on growth. The different carbohydrates tested were glucose, fructose, galactose, arabinose, mannose, sorbose, sucrose, maltose, lactose, raffinose, starch, myo-inositol, mannitol and sorbitol.

Out of the monosaccharides tested, only glucose and fructose supported good growth. Among di, tri and polysaccharides, sucrose, maltose and starch supported growth but none of the sugar alcohols used supported callus growth. Increasing concentrations of these carbohydrates glucose, fructose, sucrose, maltose and starch also increased the growth as measured by fresh and dry weight and also the accumulation of total and reducing sugars.

Carbon source is the invariable constituent of culture media used for growing plant tissues. The carbohydrate nutrition of plant callus and organ cultures has an important bearing in understanding their metabolism. The effects of certain carbon sources on the growth of tissue cultures have been reviewed by Gautheret (1959), White (1951), Goldsworthy (1964), Street (1966, 1969) and Maretzki et al. (1974). The
usefulness of sugar as carbon source may vary with its quality and quantity for the cultivation of tissues from a variety of plant species. Gautheret (1941) determined optimum sucrose concentration for the growth of carrot root callus cultures. Later (Gautheret, 1945) equimolar concentrations of other sugars were used and their nutritive value was compared with that of sucrose. Most of the plant tissues grow successfully on either sucrose, glucose or fructose but they may vary widely in their ability to use other carbon sources. Maltose has been reported to support growth of plant tissue by several workers (Simpkins et al., 1970; Anstis and Northcote, 1973). Different species of the same genus (Mathes et al., 1973), different cultivars of the same species (Chong and Taper, 1972), different organs of the same plant (Mathes et al., 1973) and even different clones derived from the same parent callus cultures (Arya et al., 1962) have been found to differ in their response to carbohydrate when grown in vitro.

Marigold tissue recorded increased weight on mannose, maltose and cellobiose; periwinkle tissue on galactose, maltose, lactose, cellobiose and raffinose; and tobacco on maltose and cellobiose (Hildebrandt and Riker, 1948). The divergence among species in their preference for different carbohydrates was demonstrated in a direct comparison of tissue isolated from five species (Hildebrandt and Riker, 1949), and the comparative effectiveness of hexoses,
pentoses, disaccharides and polysaccharides for callus growth of different species, including those isolated from tumor tissue, has been more generally reviewed (Gautheret, 1959). Galactose has been also shown to promote growth of many cultured species (Mathes et al., 1973; Meryl Smith and Stone, 1973; Thorpe and Laishley, 1974; Nickell and Maretzki, 1970), but mannose utilization is usually poor (Mathes et al., 1973; Meryl Smith and Stone, 1973; Nickell and Maretzki, 1970). In the present study galactose, arabinose, mannose, sorbose, lactose and raffinose failed to support growth of cotton callus. On the other hand, lactose and raffinose have supported growth and proved a suitable energy source for some species as reported by Mathes et al. (1973), Thorpe and Laishley (1974) and Nickell and Maretzki (1970).

Natural polysaccharides are generally not utilized by plant tissues although Hildebrandt and Riker (1949, 1953) have reported utilization of pectin, dextrin and soluble starch by tumor culture of *Catharanthus roseus* and *Chrysanthemum frutescens*. Soluble starch was found to be effective as a carbon source for virus tumor tissue of *Rumex acetosa* (Nickell and Burkholder, 1950; Brakke and Nickell, 1955), corn endosperm tissue (Straus and La Rue, 1954), callus cultures of *Rubus* (Karstens and Cats, 1960) and *Juniperus* (Constabel, 1960, 1961, 1963). In the present study, it was found that cotton callus has the capacity to utilize exogenously supplied starch to a considerable extent. There
is evidence that utilization of starch and maltose by tissues in culture is dependent upon the secretion of the appropriate hydrolytic enzymes into the culture medium (Street, 1969; Jaspars and Veldstra, 1965; Maretzki et al., 1971; Brakke and Nickell, 1951).

Sugar alcohol myo-inositol has been shown to be essential for spruce tumor tissue (Risser and White, 1964), Fraxinus callus cultures (Wolter and Skoog, 1966) and Convolvulus arvensis cells (Earle and Torrey, 1965). Hendre et al. (1975) reported that growth of maize, wheat, rice and sorghum was not significantly affected when its concentration was varied from 0 to 200 mg/l. However, cotton callus tissues failed to grow on inositol, mannitol and sorbitol at the concentrations tried. Chong and Taper (1972) have reported that callus tissues of apple could be successfully grown on sorbitol as the sole carbon source. The growth rate on sorbitol was equivalent to, and in some cases greater than, that on sucrose. Mannitol, however, is usually either not available for growth (Homes, 1967; Goris, 1971), or sustains only poor growth (Hildebrandt and Riker, 1949).
Physiological studies with Amylase, Invertase, MDH, G-6-PDH and FDPA as well as sugars and starch during growth of callus tissues of tobacco and cotton

Metabolic changes associated with wounding or ageing of plant organs have been investigated by many authors. However, very few details are available with higher plants which picturise metabolic events, the extent and timing of enzyme synthesis and their activities during growth of callus in culture.

The results obtained while following the carbohydrate metabolism in tobacco and cotton tissues during growth in culture are described in Chapter III, Section C-I, Expts. 10-11. The callus tissues of tobacco exhibited only lag and exponential phases of growth, the stationary was absent in the culture regime provided. Throughout lag and exponential phases of growth, specific amylase activity declined. Contrary to this, invertase activity per unit protein basis increased during lag and early exponential phases of growth. However, the enzyme activity declined during late exponential phase of growth. The specific MDH activity was highest during the lag phase. Later on it declined. Likewise activities of G-6-PDH and FDPA per unit protein basis shot up during lag phase, but declined during the exponential growth phase.
As in the case of tobacco, in cotton also both lag and exponential phases of growth were observed, the stationary phase being absent. All the enzymes studied: amylase invertase, MDH, G-6-PDH and FDPA showed higher activities during the lag period. The activities either remained steady or declined during exponential growth phase.

In both the tissues, total and reducing sugars accumulated fast during lag period but declined thereof very fast. Total starch accumulated in tobacco during lag period, but in cotton it declined during the same period. During exponential and post-exponential phases of growth starch content slightly declined in tobacco but it increased in cotton.

Few investigations have reported activity levels of enzymes during the culture cycle of cell and tissue cultures from higher plants. Such studies have showed that the activities of enzymes during the lag, exponential and stationary phases varied. During the growth cycle of Paul's Scarlet Rose, sucrose was hydrolysed and by the end of the exponential phase only reducing sugars were present in the medium (Nash and Boll, 1975). The reducing sugars were found to be glucose and fructose in equal amounts. A similar inversion of sucrose has been observed during the growth of tomato roots (Weston and Street, 1968), tobacco callus (Thorpe and Meier, 1973) and Acer suspension
cultures (Simpkins et al., 1970). The inversion of sucrose has been attributed to surface-located hydrolytic enzymes (Weston and Street, 1968) and more specifically to a cell-wall bound invertase (Thorpe and Meier, 1973). Wall bound invertase generally occurs in higher plants. Its activity can fluctuate considerably and seems to depend on the stage of development of the tissues in which it is found (Lyne and ap Rees, 1971; Jaynes and Nelson, 1971; Shannon and Dougherty, 1972). Moreover, Klis and Hak (1972) have shown that the activity of acid invertase in cell walls of Convolvulus callus increased markedly upon the transfer of tissue fragments to fresh nutrient medium.

Clearly the higher activities of amylase and invertase in the present study account for the large amounts of reducing sugars during the lag phase of growth. The high levels of reducing sugar in sucrose medium during the initial growth phase in rose cells similarly suggest high invertase activity (Nash and Boll, 1975).

The MDH activity both in tobacco and cotton callus cultures markedly increased in the first few days when there was hardly any growth at all. Studies with Nicotiana (De Jong, 1967) and Paul's Scarlet Rose (Nash and Davies, 1975) cell suspension cultures have shown a rapid rise in total MDH activity till the mid-exponential phase of growth followed by a very rapid decline during the remainder of
the exponential period. In the present study, however, the situation varied with the tissue; for in tobacco callus the MDH activity was high during the lag phase, while with cotton callus it was high during the lag as well as exponential period and then declined during the post-exponential phase. Givan and Collin (1967) have demonstrated high respiratory rate during the lag phase of growth in Acer pseudoplatanus cell suspensions. The respiratory rate declined with the progress of growth as also did cellular nitrogen content. Thus the energy required for exponential growth is produced prior to its onset both in case of tobacco and sycamore.

Some authors have further shown that the growth of plant cells either as callus (Komamine et al., 1969; Thorpe and Laishley, 1973) or in liquid suspensions (Fowler, 1971; Givan and Collin, 1967) is accompanied by not only in total respiratory activity but also in the absolute and relative capacities and activities of the EMP pathway and pentose phosphate pathway (PPP). Work with batch suspension cultures suggested that the enhanced respiratory activity during the lag phase of culture was characterised by an increased PPP activity relative to the EMP pathway. Thereafter during the exponential growth the respiratory activity per cell declined and so also the absolute capacity and relative activity of the PPP. Marked changes were noted
during growth in the activities of key EMP pathway and PPP enzymes and also in the level and pattern of oxidative phosphorylation (Wilson, 1971).

In the present study peak values of G-6-PDH activity were noticed during early exponential and lag phases of growth respectively in tobacco and cotton tissues. Similar activity for the enzyme FDPA was also observed in both the tissues. These findings support the earlier work of Fowler (1971), and Wilson (1971) who suggested that lag phase is characterised by intensive metabolic activity and that the balance of metabolism is directed towards biosynthesis which required the development of reducing power generated through the PP pathway. Shimizu et al. (1977) working with Acer pseudoplatanus batch cultures also supported similar view.

C - II. Physiological studies with GOT, ME and PEPC and total and reducing sugars during growth of callus tissues of tobacco and cotton in the dark.

Of the many physical factors that influence growth and differentiation, light plays a vital role. Very few physiological studies have been hitherto made with callus cultures grown in the dark. In the present study, the role of dark fixation of CO₂ which utilizes phosphoenolpyruvate derived from carbohydrate as a substrate is examined during growth.
in culture of tobacco and cotton tissues.

The control of intracellular pH in a cell may involve the regulation of intracellular organic acid levels, and Davies (1973) has suggested that phosphoenolpyruvate carboxylase activity lowers the intracellular pH by generating organic acids through a process of dark CO$_2$ fixation. He further suggested that malic enzyme by catalyzing the decarboxylation of malate has an opposite effect. Supporting the work of Davies, Bown and Lampman (1971) demonstrated the presence of these two enzymes in Avena sativa coleoptiles. Later Bown et al. (1974) showed the stimulation of Avena coleoptile growth by CO$_2$, and a correlation between malate accumulation and IAA-stimulated growth was revealed by Haschke and Luttge (1975). In addition, two recent papers demonstrated IAA stimulation of CO$_2$ fixation after a lag period of 60 minutes (Haschke and Luttge, 1977; Dymock et al., 1977), indicating that promotion of CO$_2$ fixation is secondary to IAA stimulation of H$^+$ extrusion and growth which occur with lag periods between 10 and 20 minutes (Jacobs and Ray, 1976; Cleland, 1976). Further, the PEP carboxylase from maize leaves was inhibited by physiological concentrations of oxaloacetic acid (Lowe and Slack, 1971).

Growth of tobacco and cotton tissues when grown in dark was reduced when compared with that in light. In tobacco
callus tissues, GOT activity per unit protein declined sharply during the lag phase of growth, but reached a peak during late exponential phase. In contrast to this, peak specific activity of GOT was obtained much earlier in cotton tissues (6 days earlier than in tobacco). Dhindsa et al. (1979) have noticed, however, peak GOT activity on day 4 in tobacco callus, the activity remaining more or less unchanged thereafter. Unlike this, in the present studies the GOT activities declined after attaining peak values in both cotton and tobacco callus.

Both malic enzyme and PEP carboxylase activities could not be detected on day 0 in tobacco and cotton tissues. Peak specific activity of ME was observed on day 3 i.e., during lag phase of growth in tobacco as well as in cotton. PEPC exhibited the peak value on day 3 in cotton callus, while in tobacco the peak was attained on day 9. All the three enzyme activities were higher in cotton callus when compared with tobacco.

CO₂ has been shown to be essential for the initiation of growth in sycamore cell cultures (Gathercole et al., 1976). During non-autotrophic carbon fixation, malate is known to act as an osmoregulatory solute in turgor-driven cell growth (Dhindsa et al., 1975); and it can, through its decarboxylation, also provide NADPH for reductive biosynthesis (Ting and Dugger, 1965; Fowler, 1974). Dhindsa et al. (1979)
have shown an increase in malate content with the growth of tobacco callus. If malate inhibits PEP carboxylase activity, the later activity should decrease with an increase in growth. This is confirmed in the present study with cotton and tobacco callus in both of which the PEP carboxylase activity declined during rapid callus growth (Expts. 12, 13). The malate inhibition of labelled bicarbonate incorporation into a labelled oxaloacetic acid derivative (Hill and Bown, 1978) demonstrated that malate inhibition was not due to a mass action effect in the assay which couples PEP carboxylase and MDH in malate production. The correlation of malate accumulation with IAA-stimulated growth in coleoptile tissue (Haschke and Luttge, 1975) suggested that the compensating mechanism may be malate production through CO$_2$ fixation. This suggestion supported the earlier observation that the maximum growth response to IAA required the presence of CO$_2$ (Bown et al., 1974). The continuous build-up of malate in tobacco callus (Dhindsa et al., 1979) seemed to indicate that malate maybe acting primarily as an osmotic solute to regulate cell expansion between successive cell divisions. Although no evidence is presented here, this interpretation is in keeping with the previously reported role of malate in cotton fibre growth in vitro (Dhinda et al., 1975) and also in stomatal opening (Allaway, 1973).
D. Hormonal effect on growth and development of Amylase, Invertase, MDH, G-6-PDH and FDPA and total and reducing sugars in callus cultures of cotton.

It is a tacit assumption that the regulation of plant growth and differentiation has its origin partly in changes in the pattern of enzyme activities. Even if this is admitted to be true, it does not greatly advance our understanding since the questions which are then raised reveal the extensive ignorance that prevails. For example, is the whole pattern of enzymes changed or just a related group, or only one? If many enzymes change, are all the changes of equal significance, or is only one enzymic event crucial to the physiological response? Is there a specific temporal order in the enzyme changes, or does the interlinking of metabolic pathway account for apparent sequences of events? There are many reports in the literature that growth substances either increase or decrease the enzymic activities. To mention a few, auxins have stimulated the activities of ATPase in oat coleoptile (Hager et al., 1971), RNA polymerase in soyabean hypocotyl (O'Brien et al., 1968), DNA polymerase in soyabean hypocotyl (Leffler et al., 1971) etc. Cytokinins are also shown either to inhibit many enzyme activities such as protease and RNase in several leaves (Kende, 1971; Srivastava, 1968) or promote enzymes like RNA polymerase, DNA polymerase in several calluses (Kende, 1971; Vajranabhaiah
and Mehta, 1977). Gibberellins are known to stimulate the activities of L-amylase (Yomo and Varner, 1971), RNA polymerase in pea stem (Johri and Varner, 1968), invertase in Artichoke tuber discs (Leaver, 1966), etc.

Every growth substance can apparently under appropriate conditions regulate the synthesis of RNA and probably also the synthesis of DNA, and in turn cell division. Also every growth substance can regulate the activity of one or more hydrolytic enzymes. Of these, an interesting group are the cell wall polysaccharides, since these have to pass through the plasmalemma in order to reach their substrates.

In the present study on cotton callus lower concentration of IAA (2.0 mg/l) stimulated both growth and the activities of hydrolytic enzymes like amylase and invertase as well as those of the EMP and PP pathway enzymes e.g. MDH, G-6-PDH and FDPA when compared with the higher concentration (5.0 mg/l).

Lower concentration of NAA (0.2 mg/l) promoted invertase and G-6-PDH activity, while higher concentration (2.0 mg/l) enhanced the activities of amylase, MDH and FDPA.

2,4-D at the two concentrations (0.2 and 5.0 mg/l) tested did not show any marked effect on amylase activity in cotton callus. Higher concentration, on the other hand, (5.0 mg/l) promoted invertase activity. MDH showed higher
activities at 5.0 mg/l 2,4-D level till day 15 and thereafter higher MDH activity was noticed at 0.2 mg/l level. In contrast, the FDPA was higher at 0.2 mg/l 2,4-D level till day 15 and thereafter it was more at 5.0 mg/l level. G-6-FDH was, however, promoted at low 2,4-D level (0.2 mg/l).

Of all the auxins tested, NAA at 2.0 mg/l level was more effective in promoting the activity of amylase; while 2,4-D at both the concentrations tested was highly inhibitory. The differential effect of NAA and 2,4-D as far as amylase activity is concerned has seldom been reported in literature.

At 0.04 mg/l kinetin amylase was considerably inhibited; whereas higher concentration (2.0 mg/l) had stimulatory effect. The higher rates of nucleic acid and protein synthesis reported to occur in washed slices of plant storage organs are probably associated with the increased levels of invertase and IAA oxidase (Edelman and Hall, 1965); though, on the other hand, there are several enzymes notably starch phosphorylase and amylase which fail to show higher activities in washed tissue (Palmer, 1968). Similarly, in the present studies with cotton callus amylase always failed to show higher activities during early stages of growth unlike other enzymes. Only towards the termination of culture growth, amylase registered higher activity. This is probably due to higher degradation of endogenous starch which is needed for the growth of the tissues. This view is substantiated further
when the endogenous level of starch was determined as mentioned earlier in the text.

Though GA did not support good growth of cotton callus, the activity of amylase was found quite high. Specific activity of amylase was higher on 100 mg/l GA containing medium than on 25 mg/l GA containing medium. It is well known that GA induces the de novo synthesis of amylase in the endosperm of barley (Paleg, 1960 a; 1960 b; Yomo, 1960; Chrispeels and Varner, 1966; Jacobsen, 1970; Jacobsen and Knox, 1970). This enzyme induction depends on RNA and protein synthesis, as determined by the use of inhibitors (Varner, 1964; Varner and Chandra, 1964; Chrispeels and Varner, 1967). Higgins et al. (1976) have found that (in the hormone-treated tissue) the level of translatable mRNA for α-amylase increased parallel with the increased rate of enzyme synthesis. However, it was not so with in vitro grown tissues of cotton, since the amylase activity was high towards the termination of culture period, but not during the initial lag phase.

Among the plant growth regulators, GA3 has been known to increase the activity of amylase. In the present study NAA was also found to stimulate the activity of amylase in cotton callus cultures at 2.0 mg/l level.

In Daucus carota (Ricardo et al., 1972) and in Convolvulus arvensis callus (Klis and Hak, 1972) only acid
cell wall invertases have been found. This acid cell wall invertase was, at least in part, tightly bound to the cell wall. In *Acer pseudoplatanus* cultures, acid as well as neutral invertases exist (Copping and Street, 1972). Although the ratio of bound to soluble enzymes shifted, depending on the phase of the culture growth cycle, both these enzymes were partly bound to cell wall fractions.

In the present study acidic, neutral and alkaline invertases, both wall bound and cytoplasmic, have been detected in callus cultures of *Gossypium hirsutum* (unpublished data); but only neutral cytoplasmic invertase is reported here, since it has been postulated that neutral invertase is predominantly in the cytoplasm. This has led to the hypothesis that the neutral invertase is responsible for sucrose hydrolysis in the cytoplasm in cells having less sugar demand and in which sucrose is being stored (Hatch and Glasziou, 1963; Bacon et al., 1965; Hawker, 1969; Ricardo and ap Rees, 1970).

Of the three auxins tested, IAA at 2.0 mg/l promoted invertase activity, while 2,4-D at 0.2 mg/l level inhibited it. Higher concentration of kinetin (2.0 mg/l) promoted neutral invertase activity in cotton callus cultures when compared to that in 0.04 mg/l kinetin containing medium. Of all the hormones tested, 2.0 mg/l kinetin gave high values. While GA at 25 mg/l inhibited the invertase activity,
100 mg/l GA promoted it considerably. The enhanced enzyme activities in all above treatments have been noticed during the initial lag or the exponential growth phase in cotton.

In *Acer pseudoplatanus* cells, high activity of both acid and neutral invertases correspond to periods of active growth and cell division (Copping and Street, 1972) and there is a similar trend in *Saccharum* sps. (Maretzki *et al.*, 1974). Their results were confirmed in the present study of cotton callus tissues where high activity of soluble neutral invertase corresponded to periods of active callus growth.

The relationship, or lack thereof, between cell wall invertase and cell growth has been discussed well by Edelman and Hanson (1971 b), Copping and Street (1972), Klis *et al.* (1974) and Thorpe and Meier (1973). Glasziou and Waldron (1964) have reported that in sugar cane stalks low concentrations of IAA increased the acid - invertase level, while higher concentrations suppressed the activity. Present studies support this view with soluble neutral invertase. However, Edelman and Hall (1965) showed that invertase was inhibited by IAA.

Glasziou *et al.* (1966) presented evidence that the auxin NAA, stimulated the development of invertase in sugar cane disks. The RNA content increased by about 50 per cent
within 24 hours of washing carrot disks and thus preceded the increase in invertase (Leaver, 1966). In the agreement of the results of Glasziou et al. (1966), present studies with cotton also showed development of neutral soluble invertase activity at low concentration of NAA (0.2 mg/l), but higher concentration (2.0 mg/l) proved inhibitory. The later response is similar to that reported for the invertase bound to the cell walls of carrot root and artichoke stem tubers (Edelman and Hall, 1964). Low level of 2,4-D (0.2 mg/l) inhibited invertase development to a considerable extent and this inhibition was not that much when the level of 2,4-D was higher (5.0 mg/l). Palmer (1968) had observed that 2,4-D was active in preventing the induction of high levels of phosphate metabolism and also invertase in plant tuber tissues (Palmer, 1968).

More detailed studies have yet to be made of the enzymic activity of soluble neutral invertase and its hormonal regulation in vitro.

Cell growth depends on carbohydrate utilization for two primary functions: formation of major cell components, and as an energy source. The assimilation of $^{14}$C-sucrose into a rapidly turned over soluble pool, and its extensive utilization for protein synthesis as well as protein turnover, was demonstrated by Steward and Bidwell (1966) in carrot cells. The balance between utilization for biosynthesis
or respiration shifts, depending on the stage of cell development. Increased rates of respiration in vivo was observed by many authors (Laties, 1963; ap Rees and Beevers, 1960; Click and Hackett, 1963) when tubers of many plants were sliced in thin disks and washed in water. Also dramatic increased rates of respiration in vitro was observed in tobacco (Bellamy and Bielesky, 1966), Acer pseudoplatanus (Givan and Collin, 1967), Rosa sp. (Nash and Davies, 1972), etc. In each case oxygen consumption increased twofold or more until the 4 to 6th day of culture. This was followed by a period of lessening oxygen demand over the subsequent 8 to 14 days. Respiration rate changes in Saccharum sp. cultures (Maretzki et al., 1974) and the activity of MDH in the present study of cotton callus are similar to those mentioned above. These similarities are likely to be more apparent than real, since growth rates depend not only on origin of the culture but also on culturing conditions. Nevertheless, the high mitochondrial activity that develops a few days after cells are transferred to fresh medium - a feature that all cell cultures have in common is significant. Also, peak respiration rate is correlated with active protein and nucleic acid synthesis preceding the phase of rapid cell division (Givan and Collin, 1967). Clearly the plant growth substances seemed to play a regulatory role in the activity of MDH in callus cultures of cotton in the present study.
In *Acer pseudoplatanus* lowest activities throughout the growth cycle were found in phosphofructokinase for the EMP pathway, and in glucose-6-phosphate dehydrogenase and transketolase for the PP pathway (Fowler, 1971). These enzymes were, therefore, considered rate-limiting. According to Maretzki et al. (1974) G-6-PDH is not a rate-limiting enzyme for the PP pathway in *Saccharum* sp. In the present study with cotton callus also G-6-PDH for PP pathway and FDPA for EMP pathway do not seem to be rate-limiting enzymes. On the other hand, there is similarity in the changes most of these enzymes undergo in their activity during the growth cycle, and these enzymes have activity peaks that coincide with those for oxygen consumption also (Komamine and Shimizu, 1975). Working with *Acer pseudoplatanus* cell suspension cultures Shimizu et al. (1977) have reported that respiratory activity per cell was highest during intensive metabolic activity. During this lag phase they found high levels of ATP, NAD and NADPH and also demonstrated the peak in energy charge of the culture. Once cell division has commenced there is a gradual change in the pattern of metabolism. Although the rate of oxygen uptake began to decline, the level of ATP was maintained, but the level of NADPH and the energy charge declined. This indicated that the balance of metabolism had altered and that the demand for biosynthetic reducing equivalents had decreased.
Present study was carried out with callus cultures of cotton and such a system is limited in its potential for regulatory studies because of the transient nature of the growth phases and the inflexibility of growth control. Such restrictions may be overcome by the use of continuous (chemostat) cultures since these systems allow the maintenance of steady states of cellular activity in a particular growth phase at a specific growth rate (Fowler, 1977).

Still, the higher activities of G-6-PDH, and FDPA in cotton callus cultures during the early lag phase or end of the lag phase are in agreement with the results of earlier workers.

As reported by Shimizu et al. (1977) the occurrence of a high level of NADPH after 4 days of culture is concomitant with an increased catalytic capacity of the PPP and an increase in activity of the PPP relative to the EMP pathway (Fowler, 1971). These observations of Shimizu et al. (1977) and the present studies with cotton callus support the view that the PPP is most active in early lag phase of growth and that its function is concerned with the production of NADPH for biosynthesis. Whether the increase in PPP activity relative to the EMP pathway as observed by Shimizu et al. (1977) in Acer cultures during the first few days of culture is brought about or not at the expense of EMP metabolic flux due to partial inhibition of phosphofructokinase by increasing ATP levels needs further study.
It is a general observation that cells with a high growth rate have a high activity of both the PP pathway and glycolysis, whereas in cells with a low growth rate the oxidation of carbohydrate is predominantly through glycolysis (Fowler, 1978). Working with chemostat cultures Fowler (1978) has found that at low and median growth rates PPP enzymes had low activities relative to equivalent glycolytic enzymes. However, at high growth rates this situation was reversed with G-6-PDH, a key pentose phosphate pathway control enzyme, having a significantly higher activity than the equivalent regulatory enzyme from glycolysis, phosphofructokinase. The increase in activities of hexokinase and G-6-PDH activity compared with pyruvate kinase may indicate that a major drainage of carbon precursors is occurring from the PP pathway, e.g. as pentose sugars for nucleic acid synthesis, so that the level of carbon flow through pyruvate kinase into the tricarboxylic acid cycle is substantially less than that entering the pathways at the hexose level.

Though few, hormonal control of glycolysis has been demonstrated in cell cultures. Relatively few studies are made on the hormonal regulation of MDH activity in vitro. Present studies with callus tissues of Gossypium hirsutum revealed that among auxins, IAA at both the levels tried (2.0 and 5.0 mg/l) had a marked stimulatory influence on
the MDH activity. Whereas NAA at 0.2 mg/l level had suppressed the enzyme activity. Kinetin at 0.04 mg/l level had a promotary effect in comparison with 2.0 mg/l level. GA$_3$ at 25 mg/l level increased MDH activity in the lag phase, but later on the activity was higher on 100 mg/l GA$_3$.

The activities of G-6-PDH and aldolase were enhanced in absence of exogenous auxin in Saccharum sp. cell cultures (Maretzki et al., 1974). Among the three auxins tested in the present study, 2,4-D was found to promote the activity of G-6-PDH. Contrary to the reports of Maretzki et al. (1974) who worked with sugarcane cell cultures, present studies indicated that in the presence of exogenously supplied auxins, G-6-PDH activity was increased. Kinetin at the two concentrations (0.04 and 2.0 mg/l) tested was not as effective as 2,4-D in cotton callus cultures. Also GA$_3$ had no significant control over the activity of G-6-PDH at 100 mg/l level, but at 25 mg/l level it had a promotary effect. In Acer pseudoplatanus cells the substitution of high kinetin (2.5 mg/l) for low kinetin (0.25 mg/l) concentration, lowered the specific activity of G-6-PDH (Bergmann, 1963). Despite a lower respiration rate, total glucose consumption remained unaffected. Therefore, Bergmann concluded that a non-oxidative pathway for glucose predominated when kinetin concentration was high. On the other hand, present studies with cotton revealed that high kinetin
(2.0 mg/l) favoured G-6-PDH activity when compared to low
kinetin (0.04 mg/l).

Cytokinins have also been reported to reduce activity
of the EMP pathway in *Nicotiana tabacum* (Bergmann, '1963)
and of the PP pathway in *Nicotiana glauca* cultures (Scott
*et al.*, 1964). Out of IAA, NAA and 2,4-D tested with cotton
callus cultures, NAA at 2.0 mg/l level stimulated considerably
the activity of FDPA when compared to the other auxins. This
stimulation of FDPA by NAA in the callus cultures of cotton
is hitherto a new report. Kinetin at low level (0.04 mg/l)
enhanced the activity during lag phase, but thereafter the
activity was higher at higher level (2.0 mg/l). Maretzki
*et al.* (1974) have reported that FDPA activity increased in
the absence of exogenous auxin. Present studies also support
this view to some extent. When compared to 25 mg/l of GA$_3$,
FDPA activity was higher on 100 mg/l GA$_3$ containing medium.
In other words, higher GA concentration had promoted aldolase
activity.

Since cotton is a fast growing tissue, both EMP and PP
pathway enzymes have a high activity as was also observed by
Fowler (1978) in sycamore cell suspensions.
E. Effect of carbohydrates on growth and development of Amylase, Invertase, MDH, G-6-PDH and FDPA and total and reducing sugars in callus cultures of cotton.

As already described, when plant cells and tissues are cultured aseptically, they cannot meet their energy requirements autotrophically since the normal functions of chloroplasts are frequently absent or blocked. Therefore, in plant cell, tissue and organ cultures, it is necessary to substitute suitable carbon sources. Plants translocate a restricted number of carbon compounds, viz. sucrose, oligosaccharides related to sucrose, and sugar alcohols. Of these, sucrose is pre-eminent (Ziegler, 1975). It seems reasonable then, that sucrose or any other carbohydrate source from which glucose or fructose can become readily available, should serve best the energy needs of cells grown in culture.

Callus cultures of many plants grow mainly either on sucrose or glucose or fructose. Very few plant cells grow on maltose and starch. In the present studies it is shown that callus cultures of Gossypium hirsutum could be grown successfully and equally well either on glucose or fructose or a mixture of the two or sucrose. Growth of the tissues was, however, relatively less on maltose and starch containing media.

Along with plant growth substances, the quality and
quantity of carbohydrate source that we supply exogenously played a pronounced role in maintaining the growth and metabolic events of the cells and tissues (Simpkins et al., 1970; Copping and Street, 1972; Maretzki et al., 1974). Though a synchronous growth or differentiation system would be ideal for proper understanding of the biochemical and physiological events of growth or differentiation (Komamine et al., 1978), present studies were carried out with cotton tissue grown as callus.

Disappearance or appearance of carbohydrates from the medium during cell growth has been determined. In Rosa sp. the total carbohydrate in the medium (initially 2% sucrose) disappeared by day 12 in culture with a low density inoculum. When a high density inoculum was used carbohydrate disappeared by day 8 in culture (Nash and Davies, 1972). Disappearance of the exogenous carbohydrates was similarly rapid in Ipomoea sp. cultures (Rose et al., 1972). In present studies with cotton there was rapid accumulation of carbohydrate during initial culture growth, and the carbohydrate content declined towards the termination of the culture period.

Working on Pogostemon cablin callus, Jones et al. (1973) found that during the first 48 hours, decrease of carbohydrate in the medium corresponded to a high rate of sucrose utilization for cell dry weight production, which was accompanied by a high MDH activity. About 50% of the
sucrose in the medium used for callus growth was converted to cell dry weight. Like in many plant cell cultures (Carceller et al., 1971), present studies with cotton callus also showed that increasing level of carbohydrate increased the dry weight of the cells. The total carbohydrate content of Ipomoea sp. cells increased continually first 48 hours (Rose et al., 1972) but in Saccharum sp. not until well into the linear growth phase. In Saccharum sp. as well as in callus of tobacco (Thorpe and Meier, 1974 b) endogenous reducing sugars initially increased, then decreased to 50% or less of their maximum value by day 10 in culture. Brown and Short (1969) showed a similar pattern for the UDP-glucose concentration in Acer pseudoplatanus cells. Cotton callus cultures studied here also followed a similar pattern.

Amylase activity was higher on 1% glucose medium than on 2 and 4%. Increasing concentrations of fructose likewise inhibited amylase activity. When a mixture of glucose + fructose was used the enzyme activity was higher at 2% glucose + 2% fructose level. Sucrose at 4% inhibited amylase activity markedly. As in the case of glucose, fructose and sucrose, maltose also at 1% level favoured amylase activity, while at 2 and 4% levels it inhibited the enzyme activity. Soluble starch when added into the medium, stimulated specific activity of amylase at 2% level
but again inhibited it at higher concentration (4% level).

Glucose, fructose and sucrose can reach the cytoplasm intact, while other di- oligo- or polysaccharides are probably hydrolysed extracellularly. Extracellular amylases are essential for utilization of starch by plant cell and tissue cultures since it is a heavy molecular weight polysaccharide. Karstens and De Meester-Manger Cats (1960), Nickell and Brakke (1950), Straus and Campbell (1963), Jaspars and Veldstra (1965), Marvin and Morselli (1971) and Maretzki et al. (1971) have reported the release of these amylases into the surrounding medium. Three to four days after inoculation of the tissue onto starch containing medium, the medium surrounding the tissue had showed white patches, probably an indication of release of amylase into the medium. Amylase activity was detected in the spent medium in the present studies also. Maretzki et al. (1974) have further observed that amylase release by *Saccharum* sp. cultures was increased by replacement of sucrose with starch. By and large, specific amylase activity was higher on 1% maltose medium than on other mono, di and polysaccharides incorporated media.

Enzyme maltase has been detected in callus cultures of cotton grown on maltose containing medium during the present investigation. Earlier Klis (1971) had reported the presence of α-glucosidase in cell wall fragments of *Convolvulus arvensis*. 
Increasing concentrations of glucose, fructose, glucose + fructose, sucrose, maltose and starch have inhibited soluble neutral invertase in callus tissues of *Gossypium hirsutum*. All these sugars at 4% level were more inhibitory than at 2 or 1% level. Glucose at 4% level especially has inhibited invertase activity in the present studies which could be attributed as feedback inhibition. The glucose effect on suppression of many catabolic enzymes in microorganisms has long been known (Epps and Gale, 1942; Monod, 1947). Formation of both inducible and constitutive enzymes (invertase) maybe suppressed by addition of glucose to the culture medium (Magasanik, 1961). In immature storage tissue of sugar cane, control of invertase level appears to be mediated through auxin which may increase or decrease the level depending on concentration, and a feedback control involving the level of sugar present in the metabolic compartment (cell cytoplasm) (Sacher and Glasziou, 1962; Sacher *et al.*, 1963). Sugars such as glucose, fructose and sucrose inhibited or reduced the invertase activity (Glasziou and Waldron, 1964 b). Present studies with cotton callus cultures has thus analogies with the glucose effect in microorganisms and also confirm the results of Glasziou and his co-workers who worked with sugarcane. Further Glasziou *et al.* (1966) have reported that glucose increased the rate of destruction of messenger RNA required for the production of invertase. This however, needs further
investigation at molecular level. In the present study with cotton callus, the inhibition by glucose, fructose and sucrose of soluble neutral invertase, however, did not appear until several days after inoculation. Contradicting the above results, Copping and Street (1972) have reported the highest invertase levels in the glucose grown Acer cell cultures. However, for both Acer pseudoplatanus (Copping and Street, 1972) and Saccharum sp. (Maretzki et al., 1974) the evidence against invertase control of cell growth is more compelling: cells grow equally well on glucose or sucrose; and either sugar elicits similar patterns of invertase activity during the growth cycle.

As was seen in the earlier experiments (Chapter III, Section D) the activities of MDH, G-6-PDH and FDPA increased immediately after transfer of the tissue and thus peak values could be seen during early lag phase or at the end of lag phase in most of the instances. These results are similar with the findings of many earlier workers (Givan and Collin, 1967; Nash and Davies, 1972; Komamine et al., 1969; Kikuta et al., 1977). Givan and Collin (1967) working with sycamore suspension cultures have reported that protein content and rate of oxygen uptake rise sharply early in the culture period, during which no significant increase in dry weight can be observed. Similarly, Komamine et al. (1969) have shown increased rate of respiration during the first
few days, when growth of carrot root cultures could not be distinctly measured. Accompanying the rise in respiration, the $C_6/C_1$ ratio also increased. As callus developed, the respiratory rate and $C_6/C_1$ ratio gradually decreased. Respiratory and glucose metabolism of cultured carrot root tissues showed remarkable increases in activity of the glycolytic - TCA pathway during the first 4-6 days of culture and the predominance of the PP pathway during callus development of carrot root explants.

Likewise Kikuta et al. (1971) have shown that callus of potato tuber starts exponential growth with a 5-day lag period, at which time marked production of DNA occurs. In the lag period, respiration is very much resistant to malonate and the activity of G-6-PDH passed its peak which suggested that the PP pathway was involved in the initial lag phase of callus development. Earlier Gibbs and Beevers (1955) had observed that immature plant tissues respired glucose to a large extent via the glycolytic pathway, and as the tissues differentiated and aged, the participation of the PP pathway was increasingly pronounced. Fowler and ap Rees (1970) supporting this view showed that carbohydrate oxidation in the relatively undifferentiated cells of the root apex was mainly via the glycolytic pathway and that differentiation involved an increase in the capacity and activity of the PP pathway.
In cotton, it appeared that both glycolytic and PP pathways were active simultaneously. Either 1 or 2% level of carbohydrate favoured the enzymic activities, but higher levels (4%) reduced the specific activities of MDH, G-6-PDH and FDPA in general. Sucrose, maltose and starch at 1% level have highly favoured MDH activity, but 4% sucrose inhibited MDH activity at least during the lag phase. Another highlight was the stimulation of FDPA activity by 1% glucose and 2% starch (Tables 23, 38). However, regulation in an isolated system lacking a source to sink relationship will always require a more cautious interpretation. It is important to note further that the metabolic activities measured in an asynchronous system would only represent an average value of that parameter in cells at various stages in the cell cycle (Komamine et al., 1978).

F. Organogenesis in callus cultures of Nicotiana tabacum L.

Experiment conducted with the callus cultures of tobacco to invoke morphogenetic responses is described in Chapter III, Section F. Earlier studies of Rawal (1979) in our laboratory on callus cultures of tobacco showed that manipulation of IAA and sucrose resulted either in the induction of roots or shoots or both. Hence, an attempt was made here to regulate morphogenesis by manipulating IAA and
sucrose. Rawal had also observed that except IAA, none of the tested growth hormones such as IBA, NAA and 2,4-D nor kinetin, MAP and adenine induced organogenesis in tobacco callus. Low concentrations of IAA (0.175-0.3 mg/l) favoured shoot differentiation, whereas higher levels (0.5-2.0 mg/l) favoured root differentiation. Still higher concentrations of IAA (3.0-4.0 mg/l) brought about complete suppression of organogenesis. The concentration of sucrose also in the medium seemed to have marked influence on organogenetic development. However, the sucrose mediated effect on organogenesis was apparently restricted to low concentrations of IAA (0.3 mg/l). Sucrose at 1% in the medium (IAA- 0.3 mg/l) did not support organogenesis; however, it induced shoot differentiation at 3% sucrose and roots at 6% sucrose levels. Manipulation of sucrose concentrations between 2-6% levels did not influence organogenesis in presence of higher levels of IAA (2.0 mg/l) in the medium; as roots were differentiated invariably. The increase in sucrose concentration at low (0.3 mg/l) as well as high (2.0 mg/l) IAA levels in the medium, considerably influenced the time taken for organogenetic responses. For instance, IAA at a concentration of 0.3 mg/l with 2% sucrose induced shoots in four weeks. With the increase of sucrose level to 3% (keeping the concentration of IAA constant at 0.3 mg/l) shoots were differentiated within 15 days. Similarly, in presence of 2.0 mg/l IAA,
2% sucrose induced roots in four weeks. However, with the increase of sucrose level to 3 and 6% in the same medium, differentiation of roots occurred within 12-15 days. A shift in morphogenetic pattern was also observed in *Citrus* callus by Chaturvedi and Mitra (1975) when Zeatin was added into the medium in place of BAP.

Mehra (1972), Kshirsagar (1974) and Kshirsagar and Mehta (1978) have demonstrated the influence of sucrose levels on the differentiation of gametophyte and sporophyte from the fern callus *in vitro*. The influence of sucrose in inducing morphogenetic shift and in reducing the time course for organogenesis in tobacco callus tissues seemed to be a very complex process indeed. This might involve many biochemical pathways and possibly part of the sucrose might be acting as an osmoticum also as shown in Chapter III, Section I. Rawal and Mehta (1981, in press) have recently observed that IAA at 0.3 mg/l in presence of 6% sucrose favoured root differentiation with higher activity of MDH than the shoot forming system on 3% sucrose medium. This clearly indicated the higher energy requirements for rhizogenesis than for caulogenesis. In the present study also this view is substantiated when sucrose is partially replaced by mannitol as an osmotic agent (Chapter III, Section I). Osmotic requirement for root differentiation is markedly higher than for shoot formation. Also, the activity of IAA oxidase was low in root forming system than in the shoot differentiating system. This seemed to suggest higher endogenous auxin requirement for root differentiation.
than for shoot differentiation. Thirdly, high sucrose levels have been already shown to support higher phenolic synthesis (Shah and Mehta, 1978; Kavi Kishore and Mehta, unpublished). Hence, a phenomena of auxin-phenolic synergism or antagonism could be envisaged. Such an auxin-phenol interaction has been observed to promote the rooting of the Rubus, but not in strawberry (James, 1979). Also high rates of shoot growth in Rubus have been demonstrated on phenolic free media (Boxus, 1974; James and Newton, 1977).

Besides being the source of energy, carbohydrate would provide more reducing power (NADH or NADPH) at higher levels, which might facilitate early organogenetic response.

Skoog and Miller (1957) had earlier reported that low concentrations of IAA favoured shoot differentiation in tobacco. Later many workers have demonstrated IAA induction of shoot formation in other systems; for instance, in Pergularia by Prabhudesai and Narayanaswamy (1974), in Anagallis by Bajaj and Mäder (1974), in Cyclamen by Geier (1977), etc. IAA induced root differentiation also has been reported from carrot (Nobecourt, 1939), Rumex (Nickell, 1952), wheat and barley (Gamborg and Eveleigh, 1968), Petunia (Rao et al., 1973), Pergularia (Prabhudesai and Narayanaswamy, 1974), Antirrhnun (Rao et al., 1976); Ipomoea biloba (Bhatt, 1977), Linum (Mehta, 1975), Ipomoea batatas (Kavi Kishore and Mehta, unpublished), etc. The callus may remain undifferentiated though showing prolific growth,
regardless of the hormones and nutrients to which they are exposed. Those showing exuberant growth are least conducive for regeneration as observed in *Citrus* (Chaturvedi et al., 1974).

Paulet (1965) found that low sucrose in combination with high auxin favoured root differentiation, whereas high sucrose, low auxin and high cytokinin, in combination, favoured shoot formation. Identical situation was demonstrated for tobacco tissue cultures also (Tran Thanh Van, 1977; Gaspar et al., 1977). Maroti and Levi (1977) working with *Dianthus caryophyllus* have demonstrated improvement in organised shoot differentiation with increasing sucrose concentrations. But present results showed that sucrose was effective in bringing about shifts of organogenetic response from shoot to root differentiation and *vice versa* only in presence of low concentration of IAA (0.3 mg/l) in the medium.

**G - I. Physiological studies with callus cultures of tobacco and cotton cultured on shoot inducing medium in light.**

The medium used for shoot differentiation in tobacco callus was MS basal supplemented with 0.3 mg/l IAA and 3% sucrose. Callus tissues of cotton were also cultured on the same medium, but no organogenetic response was observed. Shoot differentiation in tobacco callus occurred between days 13 and 15. Both in cotton and tobacco tissues the parameters examined during 15 day culture period were growth, accumulation of sugars and starch, and the activities
of amylase, invertase, MDH, G-6-PDH and FDPA. The elucidation of the regulatory mechanism of differentiation can be more effectively accomplished if information concerning the physiology of the cells which participate in organogenesis becomes available. Keeping this in view, on day 15, non-shoot forming tobacco callus was also analysed for the above parameters and a comparison was made with that of the shoot forming portion of the callus. In cotton callus cultures, lower portion of the callus on day 15 was analysed and compared with that of the activities of enzymes measured from upper portion of the callus.

The most dramatic change in shoot-forming tobacco callus was accumulation of starch prior to organogenesis. This starch got depleted during shoot induction. On the other hand, in the non-shoot forming portion of tobacco callus total starch content was more on day 15. In callus tissues of cotton, starch accumulated till day 15, unlike shoot forming part of tobacco callus. Starch accumulation could be a consequence of either increased synthesis of it or decreased degradation or a combination of the two. Starch accumulation has been a conspicuous feature; in other organogenetic processes both in vivo and in vitro e.g. the initiation of cotton embryos (Jensen, 1963), the induction of floral parts in cauliflower (Sadik and Ozbun, 1967), the growth of potato buds following the breakage of
of dormancy (Marinos, 1967) and in developing cotton leaves in vivo (Chang, 1979). Jensen (1963) had noticed a decline in stored starch during the development of cotton embryos. During cotton leaf development in vivo (Chang, 1979) starch accumulation was characterised by an initial rise to a maximum at the second to the fourth leaf from the apex. Then, starch content progressively decreased with leaf age. From the above literature including the present investigation, it seems that the accumulation of starch functions in the initiation of organised structures in plants. The physiological significance of starch accumulation in organ initiation, reflects the high energy requirement of the organogenetic process. As a source of energy, starch possesses a distinct advantage. The degradation of starch results in high yields of glucose-1-phosphate, the subsequent catabolism of which through glycolysis produces substrate adenosine triphosphate, without the expenditure of existing high-energy phosphates.

The degradation of starch during organogenesis has been studied in the present investigation by examining the activity of amylase during shoot formation in tobacco callus. Peak amylase activity in tobacco was noticed prior to organogenesis, whereas in cotton it was attained on day 21. Several folds higher activity of amylase was noticed in shoot forming callus when compared to the non-shoot forming portion of the callus. Saka and Maeda (1973, 1974) examined the activities
of some hydrolytic enzymes during shoot formation in rice callus. After 10 days in culture, the activity of amylase in shoot-forming regions was higher than in the corresponding callus regions. They also examined α-amylase isozymes and found the formation of a new band during shoot development. Increased activity of starch degrading enzymes during organogenesis in tobacco was also recorded by Thorpe and Meier (1974 a).

Activity of invertase increased prior to organogenesis in tobacco callus studied here. In cotton callus tissues, during the corresponding period activity of invertase either declined or remained steady. As mentioned earlier, the organ initiation process is a high energy requiring one, and during this the degradation products of starch and sucrose must be utilized. Also a continuous supply of free sugars from the medium was required for shoot formation in tobacco as shown by Ross et al. (1973) and Thorpe and Meier (1974 b).

Shoot formation has been shown to be concomitant with high rate of respiration. More pronounced MDH activity prior to shoot formation was noticed in tobacco; whereas, in cotton during this period activity declined considerably. Jensen (1963) had also observed a continued increase in oxygen uptake with the development of the cotton embryo in vivo. A parallel situation was observed in shoot-forming
and non-shoot-forming tissues of tobacco by Thorpe and Meier (1972). This is a clear indication that the accumulated starch (prior to organogenesis) is serving as a readily available reserve source of energy for the organogenetic process.

Support for this idea was obtained when glucose oxidation and the relative contribution of Embden-Meyerhof-Parnas and Pentose phosphate pathway (EMP and PPP) therein was examined. In the days preceding shoot development, tobacco tissues exhibited higher activities of G-6-PDH and FDPA; while cotton callus tissues showed significantly lesser activities during this period. When non-shoot-forming portion of the tobacco callus was observed, activities of the above enzymes were several folds lower than that of the shoot forming tissue. Changes in glucose catabolism during aging and differentiation of plant tissues have been reported by several authors (Gibbs and Beevers, 1955; Luštinec and Pokorná, 1962; Morohashi et al., 1965; Higuchi and Shimada, 1967a, 1967b; Fowler and ap Rees, 1970). Ashihara et al. (1974) had found decrease in the activity of the glycolytic pathway with aging and differentiation in hypocotyls of Phaseolus mungo seedlings. They had suggested that this might be due to the decreasing glycolytic enzyme activities and NADH content. They had further noticed that relative activity of the PP pathway in glucose catabolism was higher.
in the immature part of the hypocotyls than in the intermediary part. This could be attributed to larger amounts of NADPH and enzymes of the PP pathway. Glycolysis would lead to the production of ATP by substrate level and subsequently by oxidative phosphorylation. In case of cotton tissues, there was a marked increase in the activity of aldolase when the starch was fed (Chapter III, Results, Expt. 24). Some, but by no means all, of the reductive biosynthesis in higher plants use NADPH (ap Rees, 1974). One role for the pentose phosphate pathway could be the provision of reducing power for lignin synthesis (Higuchi and Shimada, 1967 a; 1967 b; Ashihara and Komamine, 1975). The said pathway provides reducing power (NADPH) for biosynthesis of other essential metabolites, like pentose for nucleic acid synthesis and erythrose for synthesis of aromatic compounds, via shikimic acid pathway (Davies, et al., 1964). Wong and ap Rees (1971) had reported that differentiation of the stele of pea roots was accompanied by increased activity of the PP pathway relative to that of glycolytic pathway. Pryke and ap Rees (1976) had noticed increased activity of the PP pathway relative to that of glycolysis during extensive lignification in Coleus explants. This was further confirmed by experiments investigating the contribution of $^{14}$C from specifically labelled (at C-1 and C-6) glucose to CO$_2$ released (Thorpe and Laishley, 1973). More recently Gahan et al. (1979) have
measured the activity of PP pathway during floral induction in *Spinacia oleraceae* and concluded that this pathway may be implicated in providing precursors for the synthetic processes invoked. This pathway is very important to plant cells, with as much as 30% of the total glucose passing through it, although its products may return to the glycolysis. Thus, any or all of the above mentioned functions of EMP and PPP might be important during organogenesis.

G - II. **Physiological studies with callus tissues of tobacco and cotton cultured on shoot inducing medium in the dark.**

The callus tissues cultured on the same shoot-inducing medium were incubated in total dark. As stated earlier, shoots were induced from tobacco tissues within a maximum of 15 days, whereas cotton callus cultures did not reveal any organogenetic response. To examine the physiological changes associated with shoot differentiation of tobacco callus and the non-shoot-forming callus of cotton the following parameters were studied: growth, accumulation of sugars and the activities of GOT, ME and PEPC.

In tobacco callus tissues, activities of GOT, ME and PEPC increased remarkably between days 9 and 15 i.e., during
the days preceding shoot differentiation. When the non-shoot-forming portion of the tobacco callus grown on the same medium was analysed on day 15, the activities of the enzymes were several folds lower than that of the shoot forming portion. In cotton callus, the activities of GOT, ME and PEPC declined between days 6 and 12 considerably and could not be detected at all on day 15.

It had been shown in earlier experiments (Chapter III, Results, Expt. 26) that during the shoot formation both the glycolytic and the pentose phosphate pathways are enhanced in tobacco callus. This reflected a need for additional reducing power (NADPH) for the high-energy requiring organogenetic process.

The role of dark fixation of CO$_2$ was examined here which is known to utilize phosphoenolpyruvate derived from carbohydrates as a substrate since the tissues were grown in dark. Malate arising from dark fixation of CO$_2$ was reported by Dhindsa et al. (1975) to act as an osmoregulatory solute during turgor-driven cell expansion. This malate as reported by Fowler (1974) might also provide reducing power through its decarboxylation by malic enzyme.

Present results showed that the capacities of enzymes involved in malate metabolism increased sharply prior to shoot formation in tobacco. The activities of the enzymes
were higher in shoot forming tissue than in non-shoot-forming tissue of tobacco and also that in cotton. Dhindsa et al. (1979) had observed a continuous increase in malate content with the increase in growth of tobacco callus, while the increase in malate content in shoot forming tissue was more dramatic, but transitory. Clearly, malate decarboxylation through NADP-malic enzyme also was taking place at a greater rate in shoot forming tissue. Therefore, the actual amount of malate which accumulated in tobacco could be less in shoot forming tissue than in non-shoot forming tissue. This is an indication that there would be a higher degree of internal CO$_2$ cycling in shoot forming than in the non-shoot forming tissue. In support of this view, shoot forming tobacco callus had been shown to metabolize ($^{14}$C) glucose at a faster rate than NSF tissue, leading to more endogenous CO$_2$ production (Thorpe and Laishley, 1973). The depletion of malate during shoot formation suggested that it might be providing NADPH for reductive biosynthesis of cellular constituents. This NADPH would arise from transhydrogenation during which net hydrogen transfer from NADH to NADP$^+$ takes place as suggested by Ting and Dugger (1965), and the series of reactions involved generate the internal cycling of CO$_2$. The breakdown of starch via glycolysis would provide PEP as substrate for dark fixation of CO$_2$. Similarly, triose
phosphate could enter glycolysis through the PP pathway and thus be an additional source of PEP. A rapid carbon flow in shoot forming tissue through malate may be generating NADPH to meet the additional metabolic requirements of the shoot-forming process. The malate reactions could be also playing a role as a pH-stat as suggested by Davies (1973). More evidence is, however, needed to support this view.

H - I. Physiological studies with callus tissues of tobacco and cotton cultured on root inducing medium in light.

The medium used for root differentiation from tobacco and cotton callus was MS basal supplemented with 2.0 mg/l IAA and 3% sucrose. The culture vessels were incubated under continuous light. Roots were differentiated in tobacco callus between days 13 and 15; but no roots were formed in callus tissues of cotton.

Starch accumulated prior to rhizogenesis also in tobacco tissues (Expt. 30). In cotton callus tissues accumulation of starch was observed till day 6, unlike day 9 in tobacco tissues (Expt. 31). Histochemical changes underlying shoot initiation in tobacco callus (Thorpe and Murashige, 1970; Ross et al., 1973) and rice callus (Maeda and Saka, 1973) have revealed heavy deposition of starch. But with respect
to root formation, no such reports are hitherto available. Present studies reveal accumulation of starch prior to rhizogenesis in tobacco, which might function in the initiation of organized structures. Accumulation preceded any observable organised development and this accumulation markedly decreased once the organogenetic process started. Perhaps this accumulation reflects the high energy requirements of the organogenetic process as discussed earlier.

Prior to root formation, amylase activity increased in tobacco tissue (Expt. 30). However, the activity dropped between days 12 and 15. This observation coincided with the accumulation of starch. Contrary to this, amylase activity was on continuous increase from day 6 till day 15 in callus cultures of cotton (Expt. 31). When non-root forming portion of tobacco callus was analysed on day 15, the activities of enzymes amylase, invertase and MDH were much less when compared to those in the root forming portions. On the other hand, the activity of enzymes G-6-PDH and FDPA was slightly higher than that in the root forming portion of callus (Table No. 45).

Unlike in shoot forming tissue of tobacco, specific invertase activity was lower than day 0 all throughout the culture period. But in cotton, the specific invertase activity was higher between days 0 and 9 and then it declined. Low activity of invertase in tobacco tissues
The MDH activity/mg protein increased from 0.26 units on the day preceding root initiation to 2.66 units on the day of root development in tobacco. In cotton callus also such an increase in enzyme activity was noticed between days 12 and 15, though no organogenetic development was observed.

Specific activity of G-6-PDH in tobacco was on increase till day 9, and then dropped between days 9 and 15. Same developmental pattern of enzyme was followed in cotton tissues also.

Prior to rhizogenesis in tobacco, FDPA activity increased several folds. During this period in cotton, specific FDPA activity was on decline.

As was noticed earlier in shoot forming tobacco callus, enzyme activities of amylase, invertase and MDH were higher in root forming tissues also prior to organogenetic development. On the other hand, G-6-PDH and FDPA enzymes showed higher activities till day 9 but then declined.

From the above observations it was clear that
organogenetic process required more energy which was drawn from accumulated starch and sugars. This was evident also when the specific activities of enzymes amylase, invertase, MDH, G-6-PDH and FDPA were observed. Pronounced enhancement of the above enzyme activities and the mobilization of starch from all parts of the tissue was thus indicative of the intense metabolic activity going on in root forming regions of the tissue. Stimulation of carbohydrate oxidation through the EMP glycolytic pathway at the time of organogenetic development was in keeping with the suggested role of starch and free sugars. This is also in agreement with the respiration data. The osmotic role for the degradative products of starch metabolism and the free sugars is illustrated under root forming conditions also elsewhere in the text (Chapter III, Results, Section I, Expt. 34).

Differentiation is a very complex process, even when viewed at the level of single cell within the plant body also. Different types of cells are characterized by different types of enzymes and structural proteins. This illustrates, perhaps somewhat simplistically, that differentiation involves differential gene expression; different genes are expressed in different types of cell. Even though specialized cells are characterized by the presence of specific gene products, many differentiated
cell are totipotent, in that they contain all the genetic information necessary for growth of a whole plant. The formation of the cap in the unicellular alga *Acetabularia* is regarded as a process of differentiation, which involves the sequential appearance and disappearance of a number of enzymes (Harris, 1974; Triplett et al., 1965), the synthesis of protein (Hammerling, 1963) and the synthesis of polysaccharides (Zetsche, 1966). It is also pointed that post-transcriptional events may regulate the expression not just of one gene, but of all the genes involved in a developmental sequence. The necessity for protein synthesis before and during the process of cap formation suggested that the messenger RNA molecules might be very long lived. This idea is more likely to be correct than that the enzymes involved in cap formation may be stored in inactive forms in the cytoplasm for long periods. As was shown the appearance of new polysaccharides and enzymes in the cap formation of *Acetabularia* in the present study also during shoot and root differentiation in tobacco callus, starch accumulated and enzyme activities increased.

The involvement of sucrose in processes as different as phloem differentiation and the regulation of plastid development (Dalton and Street, 1977) illustrates how the effect of a particular compound may vary according to the type of cell in which the compound is acting. The effects
of compounds such as sucrose and auxin are clearly affected by other factors. One such factor is the cellular environment. Single cells grown in suspension culture do not differentiate (Street, 1973). Addition of auxin and sucrose to the growth medium never induces the formation of xylem or phloem cells in a population of single cells; whereas, as described in the Introduction (Chapter I), these substances cause the induction of vascular bundles in callus tissues. Contact with other cells is therefore, a prerequisite for differentiation. This is another indication that cells are sensitive not only to particular concentrations of growth substances and nutrients, but also to gradients of concentrations of these substances. A growth substance can have a wide variety of effects, the actual effect being dependent on the type of cell. The same is true of the nutrients, such as sucrose, which affect patterns of differentiation. The effects of growth substances and nutrients on gene expression are thus modified by previous gene expression. Thus the involvement of cellular interactions and the developmental backgrounds of cells in the regulation of differentiation illustrates the complexity of the process of differentiation.
Physiological studies with callus tissues of tobacco and cotton cultured on root inducing medium in the dark.

The callus cultures were incubated on the same root inducing medium, but under continuous dark. Roots from tobacco callus were differentiated between days 13 and 15; but no roots were formed in callus tissues of cotton.

The appearance of roots from callus tissues of tobacco on day 15 was preceded by a pronounced increase in specific activities of GOT, ME and PEPC. In non-root forming portions of the same callus grown on the same medium, the activities of the above enzymes on day 15 were several folds lower than that in the root forming portions of the callus.

Contrary to these results, the activity of GOT in cotton increased to peak value by day 3. Thereafter the activity decayed till day 15. Malic enzyme and PEPC could not be detected on day 15 in cotton callus cultures, while they increased during the same period in tobacco tissues.

As shown earlier during the formation of shoots from tobacco tissues grown in the dark, activities of the enzymes GOT, ME and PEPC also increased during rhizogenesis; the non-root-forming tissue showing much less activity of all these enzymes.
To meet special metabolic requirements of the root-forming process, like the earlier discussed shoot-forming one, a rapid carbon flow through malate may be generating NADPH. Increase in the level of NADPH, ATP and energy charge were also observed in the initial period of culture when the cell division is maximum (Shimizu et al., 1977). Continuous build-up of malate in non-shoot-forming tobacco tissue (Dhindsa et al., 1979) provides evidence for such a view. Malate in non-organ forming tissues may be acting primarily as an osmotic agent to regulate cell expansion between successive cell divisions as shown in cotton fibre growth in vitro (Dhindsa et al., 1975) and also during stomatal opening (Allaway, 1973).

As shown earlier (Chapter III, Results, Section H - I, Expt. 30) accumulation of starch prior to organogenetic development and its degradation during the process of differentiation to oligosaccharides could increase the osmotic potential of the cells resulting in increased water uptake. The latter would keep proteins fully hydrated and allow maximum enzyme activity. Thus if, as it appears, both reducing power and osmoregulation are needed for organogenesis, tobacco callus utilized alternative pathways for fulfilling each of these requirements.
I. Osmotic requirement for shoot and root formation in callus cultures of tobacco.

Chapter III, Section I, Expt. 34, described the effect of mannitol as an osmotic agent and possible osmotic involvement in organogenesis in callus tissues of tobacco. The reason for selecting mannitol as an osmotic agent being (i) mannitol does not support tissue growth in tobacco (Hildebrandt and Riker, 1949); (ii) it has been demonstrated that mannitol is not metabolised by tobacco (Trip et al., 1964); (iii) (though) it does enter callus tissues of tobacco (Klenowska, 1971). Thus potential separation of an endogenous osmotic requirement from an exogenous medium-imposed one could be possible.

Mild osmotic stress not only increased callus growth but also modified the cellular morphology in tobacco (Klenovska, 1973, 1976) and soybean tissue cultures (Kimball et al., 1975). The importance of osmotic conditions in the culture of zygote embryos of Datura (Rietsema et al., 1953), Capsella (Raghavan and Torrey, 1963) and Hordeum (Granatek and Cockerline, 1978) have also been documented. Doley and Leyton (1970), Granatek and Cockerline (1978) have outlined osmotic-hormonal interactions. Maretzki et al. (1972) have reported furthermore that small changes in somotic stress lowered reducing sugar concentrations and a decrease in invertase activity in sugar cane cell cultures.
The results in Section I and Table 49 suggested that mannitol alone could not give any organogenetic response. Also 1% sucrose with 0.3 mg/l IAA could not evoke any morphogenetic response. Shoots were formed only when 1% sucrose was supplemented with mannitol to give the molar sucrose equivalent to 2%. The dual role of sucrose as an osmoticum and an energy source was indicated by the inability of mannitol to completely substitute for sucrose below the 1% sucrose level. This 1% sucrose level maybe the saturating level for carbon flow through the cell membrane and energy-producing pathways, but an additional 1% sucrose (i.e., total 2% sucrose) was needed to produce the optimum level for turgor generation during shoot formation.

Shoot formation was promoted from 50 to 65% frequency and from solitary to 7 to 9 shoots per callus mass when 2% sucrose was supplemented with mannitol to give the molar sucrose equivalent to 3%. This promotion of shoot formation by mannitol was not a growth response as shown in Table 49. The failure of mannitol to replace the sucrose requirement for growth is consistent with the findings that there are different patterns of malate accumulation by dark fixation of CO₂ during growth and organogenesis (Plumb-Dhindsa et al., 1979). Their results confirm that malate does not accumulate during shoot formation but it does during callus
growth. Studies of Dhindsa et al. (1975) on cotton fiber indicated that levels of potassium and malate in fiber were highest when the rate of fiber growth was maximum and this accumulated malate fulfilled osmotic requirement for growth. This might be true even for tobacco. It is common knowledge that the degradation products of the accumulated starch and free sugars from the medium are important at the site of organ formation.

It was obvious from the present results that osmotic requirements for shoot and root formation are different (Table 49). Number of roots also increased per callus mass with the increase of mannitol content. The success in partially replacing the sucrose requirement for shoot and root formation with mannitol in osmotically equivalent levels supported the view that part of the sucrose is acting as an osmoticum (Thorpe and Murashige, 1970; Barg and Umiel, 1977; Thorpe, 1977).

These results are also consistent with the hypothesis of Cleland (1967, 1971, 1977) and Zimmerman (1977, 1978) for turgor-driven growth and cell expansion. According to this view, high levels of sucrose and mannitol in the medium would act against the creation of a critical turgor pressure which must be established before cell expansion can occur. This condition of stress would inhibit callus growth, shoot and root formation. This was also evident from the studies
of Dhindsa et al. (1975) during cotton fiber extension by extracellular osmotic agent. Similarly, promotion of shoot and root formation by mannitol, in presence of adequate sucrose as energy source, may reflect the generation of a turgor which results in healthy root and shoot formation.

It was clear from these studies that sucrose was not solely acting as a source of energy and carbon during formation of root and shoot in tobacco callus but also providing appropriate osmoticum. Secondly, osmotic requirements for root and shoot formation were different. The present investigation has, however, no parallel studies in my knowledge, to substantiate this observation.

J. Effect of an inhibitor (Rifamycin) on organogenesis and carbohydrate metabolizing enzymes in callus cultures of tobacco.

Reich et al. (1961) have reported that small amounts of the antibiotic actinomycin D when applied to a tissue culture inhibited RNA synthesis, while synthesis of DNA and protein, at least initially, was not affected. The inhibition may be on enzyme systems and in several cases may be caused by structural similarities between the normal constituents and the antibiotic.

Rifamycin is a specific RNA polymerase inhibitor in
bacteria, since when applied mouse RNA polymerase was not affected. But evidence exists that in some plants it has activity. Rifamycin is reported to inhibit only the free enzyme before its attachment to DNA, and if this has already occurred and if the nucleotides have been added, the enzyme is protected against the action of these antibiotics. Earlier work with rifamycin indicates that its inhibition is at a very early stage in the process of transcription; if applied once polymerization of the nucleotides has commenced, it has no effect (Leshem, 1973).

Two concentrations of rifamycin (0.01 and 0.1 mg/l) were added into the standard growth medium of tobacco, and growth was slightly inhibited at 0.1 mg/l rifamycin level. Activities of amylase, invertase and MDH were not changed much, while G-6-PDH and FDPA were many folds higher on 0.01 mg/l rifamycin containing medium than on 0.1 mg/l level. However, growth as well as activities of enzymes tested were several folds higher on the standard growth medium (control) than on rifamycin containing media.

When incorporated into the shoot inducing medium of tobacco rifamycin at lower concentration (0.01 mg/l) delayed the organogenesis from two to four weeks. Also it reduced frequency of response from 70-75% to 50-60%. At higher concentrations of rifamycin (0.1 mg/l), organogenesis was further inhibited with a frequency response of only 5-10%.
Activities of the enzymes tested were higher at 0.01 mg/l rifamycin containing medium than at 0.1 mg/l. When compared with shoot forming medium (control) of tobacco, activities of these enzymes were much suppressed on rifamycin containing media.

When rifamycin (0.01 and 0.1 mg/l) was added into root inducing medium of tobacco, root differentiation was completely inhibited. Secondly, rifamycin at 0.1 mg/l level markedly inhibited activities of the enzymes amylase, invertase, MDH, G-6-PDH and FDPA. In comparison with normal root inducing medium of tobacco (i.e. control), activities of the above mentioned enzymes were inhibited on rifamycin containing media.

The developmental programme of a cell involves, among other things, the appearance and disappearance of specific times of specific proteins. Rifamycin delayed the shoot formation. Is the different concentrations of IAA in the shoot and root inducing media and their influence on RNA polymerase responsible for this differential effect of rifamycin needs to be further studied.

If the effect of rifamycin is on RNA polymerase, then inhibition of protein synthesis should be encountered almost immediately. Although it is widely assumed that much gene expression is regulated at the transcriptional
level, there is very little evidence to support this assumption. Apparently, the control of the expression of a number of genes is at the translational and post-translational levels. However, it is not yet clear whether translational and post-translational control are more important than transcriptional control, as has been suggested by Harris (1974).

The available evidence indicates that messenger RNA molecules in the cytoplasm of plant cells are long lived. A rapid decline in the activity of a particular enzyme must therefore, be achieved by one or more of the following methods:

(a) Increasing the rate of degradation of the messenger RNA; detected by changes in enzyme activities, and by changes in the structure and content of organelles and membrane components. Further, different types of cells are characterized by different types of enzymes and structural proteins. This illustrates, that differentiation involves differential gene expression: different genes are expressed in different types of cell. Inhibitors of protein synthesis and RNA synthesis when used carefully, can give general information on the control of gene expression. For instance, actinomycin D (inhibitor of RNA synthesis) at concentrations that completely inhibit seedling growth, does not prevent the increase in the activities of isocitrate lyase and malate
synthetase in the cotyledons of *Citrullus* (Hock and Beevers, 1966). Similarly, 6-methyl-purine, at concentrations which abolish RNA synthesis, fails to prevent induction of nitrate reductase in *Chlorella* (John et al., 1973). In the present study it is seen that rifamycin at higher concentrations (0.1 mg/l) is more effective in inhibiting enzymes such as amylase, invertase, MDH, G-6-PDH and FDPA. It is thus likely that the increases in activities of enzymes of *Citrullus* and *Chlorella* are not dependent on the synthesis of messenger RNA, and control is therefore, post-transcriptional. Whereas in *Nicotiana tabacum* the inhibition of some of the carbohydrate metabolizing enzymes by rifamycin indicate that the activities of these enzymes are dependent on the synthesis of mRNA,

(b) Inactivation of the messenger RNA,

(c) Increasing the rate of degradation of the enzyme. The reduction in nitrate reductase activity under non-inducing conditions is prevented by cycloheximide in *Hordeum vulgare* (Travis et al., 1969). Similar reductions in the rate of decline of activity in the presence of inhibitors of protein synthesis have been reported for a number of other enzymes. Present physiological studies with rifamycin on callus cultures of tobacco during growth and organogenetic development also indicate that protein degradation as
well as protein synthesis must be taken into account when considering the control of gene expression.

(d) Inactivation of the enzyme.
The existence of specific inhibitors may cause inactivation of the enzyme phenylalanine ammonia-lyase as shown by French and Smith (1975) and deoxyribonuclease in Chlorella by Schonherr et al. (1970). The possibility of inhibition of the enzyme activities by rifamycin in tobacco callus cultures could not be ruled out by inactivation of the enzyme also as shown above.

On taking cognizance of the entire data obtained through experimentation in the present study, the following observations come into focus:

1. In both tobacco and cotton callus tissues during growth, the lag phase of the culture period was characterized by an increasing invertase activity, respiratory activity (MDH) and also more activity of PPP relative to the EMP pathway enzymes.

2. When the above tissues were grown in the dark, there was a decline in growth values. The enzymes GOT, ME and PEPC showed higher activities during lag phase of growth than in the subsequent growth phases.

3. Auxins, cytokinins and GA₃ greatly influenced
the enzyme activities of amylase, invertase, MDH, G-6-PDH and FDPA. Of particular interests are the findings that NAA caused pronounced enhancement in the activity of amylase and GA$_3$ that of FDPA.

4. Higher concentrations of carbohydrates such as glucose, fructose, glucose + fructose, sucrose, maltose and starch inhibited enzymes such as invertase in cotton tissues. In cotton, both the glycolytic and PP pathways were equally active. While lower concentrations of the above sugars favoured or promoted many enzymes, higher levels (4%) suppressed the activities of MDH, G-6-PDH and FDPA in general.

5. The most dramatic difference between organ forming and non-organ forming tobacco callus was the accumulation of starch prior to organogenesis. This starch disappeared subsequently and was presumably utilized during the initiation and development of shoot or root primordia. Starch accumulation in cotton tissue was less conspicuous, and it was not mobilised till the termination of culture period.

6. The utilization of starch involved enhanced rates of degradation of the metabolite during organogenetic process as revealed by high activity of amylase.

7. The activity of invertase also increased markedly
in the shoot and root forming tissues, than in the non-organ forming tissues of tobacco and cotton. A continuous supply of free sugars appeared to be essential for morphogenesis.

8. The accumulated starch and free sugars probably might be serving as a readily available reserve source of energy for organ generating processes. Evidence in support of this idea was obtained when the activities of respiratory enzymes in shoot-forming, root-forming and non-organ forming tobacco and cotton callus tissues were compared. Higher activity of MDH was observed during shoot and root formation in tobacco callus tissues. MDH activity was by and large substantially higher during rhizogenesis than during caulogenesis. These results further indicated that requirements for energy (ATP) and reducing power (NADH or NADPH) were higher for root formation than for shoot differentiation.

9. Examination of glucose oxidation further confirmed the above findings. Increased activities of enzymes of both the EMP glycolytic and PP pathways of glucose oxidation were found in organ forming tobacco callus than in non-organ forming tobacco and cotton callus tissues. Stimulation of carbohydrate oxidation through EMP pathway during organogenesis was in keeping with the suggested role of starch. This glycolytic pathway
would lead to the production of ATP at substrate level and subsequently by oxidative phosphorylation. PP pathway is significant in terms of reducing power generation for biosynthesis as well as for the production of highly reactive intermediates for the synthesis of essential cell constituents like nucleic acids, etc.

10. Furthermore, the non-autotrophic CO₂ fixation was markedly higher during organ forming tissues of tobacco as determined by the activities of malate metabolism enzymes, viz. PEPC, ME and GOT. Perhaps malate must be generating reducing power in the organ forming process.

11. From the experiments conducted with mannitol as an osmotic agent in tobacco callus tissues, it is inferred that sucrose was not acting solely as a source of energy and carbon during organ formation; but also providing suitable osmoticum. Secondly, osmotic requirements for root and shoot formation were different.

12. Rifamycin, a specific RNA polymerase inhibitor delayed shoot formation, while it completely inhibited rhizogenesis in tobacco callus cultures.
From the present study it becomes quite apparent that organogenesis from unorganised cells is under strict gene control and is accompanied by the synthesis of definite functional proteins. It will be worthwhile to examine the spectral changes in the isoenzyme patterns of various enzymes involved in starch and sucrose metabolism, enzymes of the EMP, PPP and TCA cycle, malate metabolism and many more. Also the changes in the molecular species of RNA cannot be ignored. As Bonner (1965) has pointed, changes in metabolism in which new enzymes originally absent are synthesized or enzymes present show increased synthesis and/or activity must a priori be a cause rather than a result of differentiation. However, this does not indicate how differentiation is regulated at the molecular level.

If we accept the totipotency of all living cells containing a normal complement of chromosomes, then one must ask why is it that certain species are so intractable in culture as in the case of cotton in the present study. This is probably that we have not yet determined the right cultural conditions or the proper additives to the medium. There is something much more fundamental which is involved, and it relates to our complete ignorance of how regulation is controlled at the molecular level. Also, how a single cell among many becomes activated remains speculative. The
role of physiological gradients can explain the formation of loci in which the organogenetic cells may be found. Formation of meristemoids from activated cells is an apparent structural requirement for organogenesis \textit{in vitro} (Thorpe, 1978). This meristemoid is initially plastic and capable of becoming either a shoot or a root primordia (Bonnet and Torrey, 1965). Perhaps it is this failure of cells to undergo complete dedifferentiation and become mitosis-determined that is at the center of the problem of regulation.

Finally, we have a long way to go before we have mapped the physiological and biochemical events associated with the shift in metabolism which occurs during organ formation. To facilitate this task, we require the development of a system in which the precise location of cells which are synchronously undergoing dedifferentiation and differentiation can be routinely observed and manipulated \textit{in situ}. Also mention must be made, that these cells should be easily removable and available in large quantities for detailed biochemical studies. Only then we can begin to look at regulation at the molecular level and attempt to answer the fundamental question of what regulates a single cell and sets it apart on the altered pathways of differentiation.

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